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"Bacterial Community Analysis in Acidic Mine Waters"

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Affidavit

I confirm/certify that I have written this work independently and I have not use any tools than those indicated. This sworn statement also refers to the illustrations.

26th September 2014 Marina Astudillo Pascual

Gratitude

First of all I would like to thank Herrn Prof. Dr. Michael Schlömann, who has provided me this interesting topic, as well as allows me to work in the Working Group on Environmental Microbiology. Also to Maria José López as my Spanish supervisor and the teacher who motivated to love the Microbiology.

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Abstract

From the discovering of first iron oxidizer microorganisms, bioleaching community has been studied by a great deal of researchers. Nevertheless, the microbial community structure involved in this process is still not clear. The increasing interest to replicate the bioleaching process for environmental bioremediation or mineral extraction has fostered this topic as the body of many studies nowadays.

Therefore, information from different researches about the most successful techniques for iron oxidizer identification have been gathered in this thesis, in order to find out a complete phylogenetic tree of the bioleaching microorganisms existing in mine waters of *Reiche Zeche*, Freiberg (Germany).

Diverse water samples from leaching have been analysed throughout different DNA identification techniques and their fingerprint obtained by ARDRA, sequenced and analysed by T-RFLP.

Expected and unexpected results have been obtained about the microbial community structure. Thus, common iron oxidizers such as *Acidithiobacillus* sp. or *Leptospirillum* sp. has been detected along with a large list of uncultivated microorganism which made up their own separated group in the phylogenetic tree. Therefore knowledge on the distribution and biodiversity of this group of iron oxidizers is still not completed making necessary deeper analyses.

Resumen

Desde el descubrimiento de las primeras especies de microorganismos oxidadores de hierro, la comunidad implicada en la biolixiviación ha sido objeto de estudio de un gran número de investigaciones. A pesar de ello, la estructura de dicha comunidad sigue sin estar clara. El creciente interés por replicar el proceso de biolixiviación para usos como la bioremediación ambiental o como técnica de extracción minera ha fomentado este tema como objetivo de muchos estudios hoy día.

En este trabajo se reúne información de distintas investigaciones sobre las técnicas más exitosas para la identificación de microorganismos oxidadores de hierro, con la intención de obtener un árbol filogenético completo de los organismos implicados en fenómenos de bioloxiviación existentes en las agua de la mina de *Reiche Zeche* Freiberg (Alemania). Diversas muestras de agua lixiviada han sido analizadas usando diferentes técnicas de identificación de ADN, como ARDRA, secuenciación y análisis T-RFLP.

Se han obtenido tanto resultados esperados como inesperados sobre la estructura de la comunidad microbiana. Así, además de microorganismos oxidadores de hierro comúnmente encontrados, tales como *Acidithiobacillus* sp. o *Leptospirillum* sp.,se ha encontrado una larga lista de microrganismos no cultivados, los cuales forman su propio grupo aislado en el árbol filogenético. Por tanto, el conocimiento en la distribución y biodiversidad de este grupo de oxidadores de hierro sigue sin estar completo, haciendo necesarios análisis más detallados.

Bacterial Community Analysis in Acidic Mine Waters

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List of abbreviations

AMD: Acid Mine Drainage

ARDRA: Amplified Ribosomal DNA Restriction Analysis

BSA: Bovine Serum Albumin (ThermoScientific, Molecular Biology, Unites States)

dNTPs: Deoxynucleotide Triphosphate

LB_medium: Lurian Bertani (Sambrook & Russel 2001)

PCR: Polymerase Chain Reaction

Rpm: Revolutions Per Minute

RZ: *Reiche Zeche*

TAE: Tris-Acetat-EDTA

T-RFLP: Terminal Restriction Fragment Length Polymorphism

1. Aim

The final purpose of this research was the identification of bacterial diversity of the acidic environment of mine of *Reiche Zeche*, Freiberg, Germany, as well as find out which of these microorganism are the possible key players of the natural bioleaching process. Additionally, a preliminary study of the favourable conditions, such as pressure, temperature, pH and geochemical conditions, for a more efficient bioleaching process was also attempted.

For this purpose water from the deep-mine was sampled and the bacterial DNA was extracted and amplified. A sequence analysis of these bacterial DNA was performed and the bacterial composition of the samples was determined. Final data was added to a Clone-Library in order to store the gathered information and make it available for other researchers.

2. Bibliographic background

"The iron-oxidizing proteobacteria" has been reviewed by Hedrich (Hedrich, 2011). This review provide a comprehensive current description about the phylogenetic and physiological diversity of the iron-oxidizing proteobacteria. In accordance with the information gathered in such review, bacteria such as *Thiobacillus ferrooxidans, Acidithiobacillus* spp. and *Ferrovum myxofaciens* are common inhabitants of extremely acid environments (about 3 and 2 pH) being named then as extreme acidophiles. *Thiomonas* spp. is the main moderate acidophile, living within an optima pH for growth of 3–5.

In addition, regarding with the article "Population Dynamics of Iron-Oxidizing Communities in Pilot Plants for the Treatment of Acid Mine Waters" (Heinze, 2009), The habitat was definitely dominated by two groups of Betaproteobacteria affiliated with species "*Ferrovum myxofaciens*" and with strains related to *Gallionella ferruginea*. The clear dominance was perceptible even under changing pH and iron concentration conditions.

Consequently aforementioned articles stablished the hints of which iron oxidizer microorganism were expected to be found in this Thesis.

3. Introduction

3.1 Geology of mine *Reiche Zeche*

The Freiberger deposit is characterised by metamorphic rocks from Precambrian to Lower Paleozoic and is composed by three mining areas called *Freiberg Muldenhütten, Halsbrücke* and *Brand-Erbisdorf* (Figure 1).

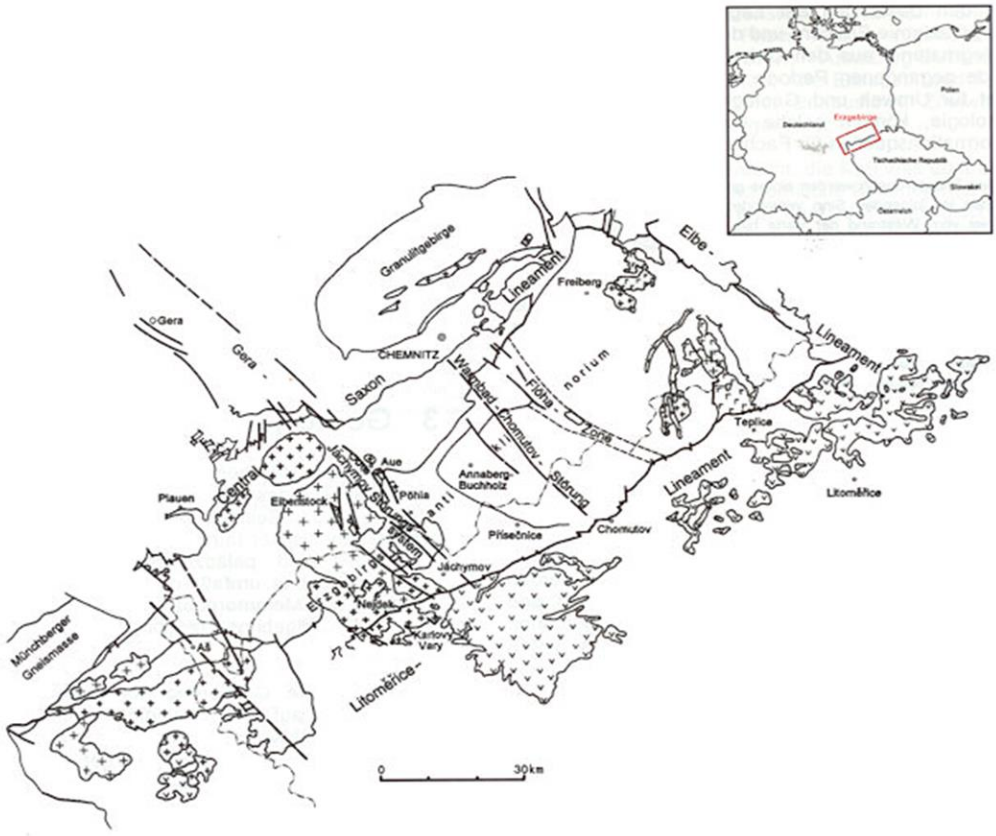


Figure 1: Freiberger deposit map

The deposit has 15 kilometres North-South extension, 9 kilometres from East to West and 320 to 500 metres over sea level. A place where the predominant material is the rock named grey gneiss.

Changings in pressure inside and diverse contractions, resulting from cooling of the outside, lead to fractures inside of the gneiss, which were filled with different minerals, what resulted in about 500 lodes in Freiberger district forming a complicated net of gaps in different directions.

After several periods of mineralisation, minerals like galena, sphalerite, pyrite, chalcopyrite, and quarz can be found in common.

3.2 Mine waters of *Reiche Zeche*

The mine waters are usually classified as acidic and metal-rich. The water coming from rivers, aquifers flowcharts and rain water “penetrates through the flooded shafts, galleries, backfilled veins and open veins and gets enriched in acid metals” (Baacke 2000). Indeed, many parts of the Freiberg mining district, especially above the flooding water level, have extremely acid mine drainage (AMD) with pH as low as 2 (Baacke 2000).

Aforementioned extremophile acid mine drainage is an environment in which the majority of prokaryotic and eukaryotic organisms could not survive, but some "acidophiles" *Bacteria* and *Archaea* thrive within it (Johnson et al., 2003) and even modify the geochemical conditions of the field.

3.3 Extremophile bacteria as bioleaching promoters and water pollution

Such "acidophiles" microorganisms mainly act over several minerals modifying their physical and chemical state. The combination of this biological activity with other abiotic reactions has given an incredible result on the mining extraction techniques field. For a better compression of how relevant could the biological activity be, it is required to have in mind determinate mineral behaviours, like sulphide reactions under extremely acidic conditions.

Sulphide minerals such as pyrite (FeS_2), commonly are chemically and biologically stable in areas lacking oxygen and water. However, upon exposure to moisture or air iron oxidation immediately starts. This causes the break of the bond between metal and sulphur releasing it as described in the general leaching reaction (Reaction 1) and the following example for the first oxidation (Reaction 2)(Johnson et al., 2003).

$$MS + 2\ Fe^{3+} \rightarrow M^{2+} + S^0 + 2\ Fe^{2+} \quad (1)$$

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O^{2-}{}_3 + 6H^+ \quad (2)$$

It is show that the bond breaking is fostered by ferric iron (Reaction 1) that reacts becoming into ferrous iron.

Ferrous iron is stable under acid conditions. Therefore in mining habitats with acid drainage, this leaching process would stop whenever all the reactant ferric iron becomes into ferrous. At this point is where the microorganism's role is crucial.

Because of the activity of the microorganisms, the ferrous iron can be oxidized to ferric iron and so the leaching process will continue (Reaction 3). This new process is called bioleaching.

$$2H^+ + 0.5O_2 + 2Fe^{2+} \rightarrow 2\ Fe^{3+} + H_2O \quad (3)$$

Indeed, bioleaching is not an isolated reaction, bioleaching involves in general three important sub-reaction, viz., and attack of the sulphide mineral by a chemical ferric leach producing ferrous iron. These reactions also take place in the abiotic leaching process. But in addiction, bioleaching process includes a microbial oxidation of ferrous iron (Reaction 3) and perhaps sulphur compounds (Reaction 4) (Hansford et al., 1999). Only a certain group of bioleaching microorganisms carry out the oxidation of sulphur, decreasing even more the pH in the area and generating acid mine drainage (AMD).

$$S^0 + 1.5O_2 + H_2O \rightarrow 2\ H^+ + SO_4^{-2} \quad (4)$$

The role of the bacteria is to re-oxidise the ferrous iron back to the ferric form and maintain a high redox potential (Hansford et al., 1999). These activities are crucial considering that ferrous iron is stable under acid pH condition. Exclusively when the metabolites produced by the bacteria create a high oxidising potential on solutions (Escobar et al., 2008) ferrous iron becomes non-stable, enabling its solubilisation and the conversion anew to the ferric form. Due to the metabolites are not generated by the abiotic bioleaching, ferrous solubilisation is not possible by the abiotic pathway in such acid mine waters (pH < 4).

That is why oxidation rate is far higher in the presence of certain iron oxidizers prokaryotes, due to the ferrous iron re-generation (Reaction 4).

Hence it has been shown that, although the abiotic oxidation may react in anaerobic as well as in aerobic environments, in the biotic oxidation oxygen presence is required (Johnson et al., 2003), only the biotic pathway is able to regenerate the ferric iron within extremely acid waters. It is for this reason that the combination of both pathways causes a highest efficiency in the mining extraction techniques field, as well as the reduction of the pH and thus acid mine Drainage (AMD) (Reaction 3 and 5) (Johnson et al., 2003)

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad (5)$$

This natural process can be used by human activity, creating a new definition in which bioleaching is considered as a simple, inexpensive and eco-friendly human technology for mineral extraction from mine. The bioleaching technology takes advantage of the natural microorganism's activity for the mineral extraction or bioremediation of contaminated soils (Bosecker, 1997). Such microorganisms are found in most mines all over the world. They feed on nutrients in minerals with the advantage that this same nutritional process causes the extraction of desired precious metals embedded in ore – like copper, zinc or indium. So to say, microorganisms are used to solubilize the mine metals (Bosecker, 1997), avoiding damaging caused by non-environmental friendly methods such as melting or roasting. This method is often used nowadays for extraction of ore with low metal concentrations (Mithra, 2014).

Iron-oxidizing *Bacteria* and *Archaea* are included in the lithotrophic prokaryotes group (''rock eating''). The most common and important *Bacteria* in this process due to their oxidising capacity are *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Escobar et al., 2008). *T. ferrooxidans* has been recently renamed *Acidithiobacillus ferrooxidans* (Johnson et al., 2003).

4. Materials and Methods

The method structure employed in this research is made up by six important steps: Gene cloning with Polymerase Chain Reaction, Terminal Restriction Fragment Length Polymorphism (T-RFLP), ARDRA, Transformation and Cultivation. The results depend directly on these six main steps. Hence, to know how aforementioned steps were carried out, their characteristics and the reasons for choosing one protocol or another becomes a highly significant aspect.

In the first case, the Gene cloning with Polymerase Chain Reaction (PCR) allows to generate millions of copies of a specific gene. For this purpose 16S rRNA gene sequences was used in this work to study bacterial phylogeny and taxonomy for several reasons here named (i) its presence in almost all bacteria; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatic purposes (Patel, 2001)

Once the PCR product is checked, the T-RFLP proceed to measure the size polymorphism of terminal restriction fragments from a PCR amplified marker, providing a diversity assessment (Bohannan., 1999).

During the investigation it was also necessary to run a "transformation" with *E.coli*, also called "cloning".

As the last method and the most crucial together with sequencing, to reach the research aim, is the ARDRA. This method includes the use of different enzymes known as restriction endonucleases. These enzymes cut DNA-fragments at specific locations based on the nucleotide sequence. DNA treatment with a restriction enzyme produces many smaller fragments, of varying sizes. These fragments are visualized in the Electrophoresis as separate bands of similar chain length. Each species is defined by a determinate bands pattern. So ARDRA allows to identify the different DNAs presence in the samples and make a first population approximation of each species gathering the ones with the same shape.

4.1 Water Sampling

The sampling place is located approximately at 50.92 º, 13.35º, and around 150m underneath Freiberg city's ground (Figure 2). Nearby this localization, seven separated and different water samples were taken in the mine of *Reiche Zeche*, from puddling water (named RZ_2, RZ_3, RZ_4, RZ_5, RZ_6 and RZ_7) to roof leaking water (RZ_1). Always trying to cover as much as possible of the area in order to maintain the representation of the mine water. For the sampling task, sterile 50 mL Falcon Tubes and 800 mL glass bottles were used. During the samples collection an *in situ* geochemical analysis of the water sample was done (Table 1.1, 1.2 and 1.3)

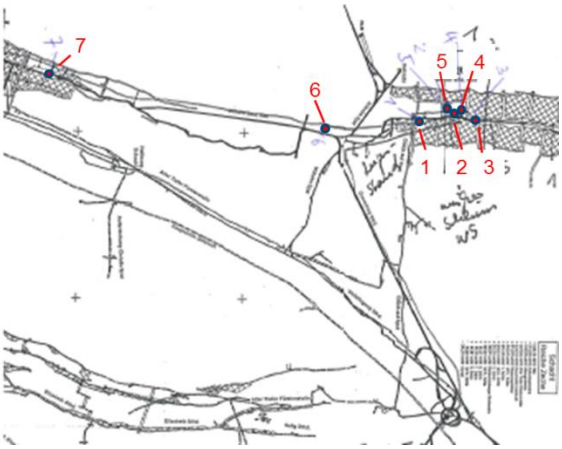


Figure 2: Sampling points, mine of *Reiche Zeche*.

SAMPLE	Water amount (L)	[Zn2+] mg/L	[Fe3+] mg/L	Fe2+ presence	[mV]±50mV Redox
RZ_1	1,2	-	2	-	210
RZ_2	1,6	32	245	Positive	600
RZ_3	2	1302	4114	Positive	640
RZ_4	1	3393	4055	-	730
RZ_5	1,1	595	2576	-	800
RZ_6	0,75	2	9	-	
RZ_7	2	7159	7696	-	710

Table 1.1: *In situ* geochemical data in the sampling points.

Table 1.1 and 1.2 and 1.3 shows the characteristics of water samples collected. Ferrous iron was analysed qualitatively (absence or presence) because the device employed for the *in situ* analysis did not have the necessary standards to quantify it (Table 1.1)

Sample	Look	pH	T [°C]	Conductivity [µS/cm]	O2-content [%Saturation]	O2-content [mg/L]
RZ_1	Colourless,clear	7,4	13,7	8	67	6.9
RZ_2	brownSlightlyturbid	2,9	13,3	11.9	47	5.5
RZ_3	brown,turbid	2,7	13,8	15.9	57	5.2
RZ_4	brown,very turbid	2,0	12,6	15.6	52	5.8
RZ_5	brown,turbid	2,5	12,7	11,7	55	5,8
RZ_6	grey-green Sediment	6,8				
RZ_7	Red-brown,clear	2,6	11,8	24,1	59	5,2

Table 1.2: In situ geochemical data in the sampling points.

Sample	Site of sampling	Hints
RZ_1	1.Level, *Wilhelmstehender*N	Dripping water from the ceiling
RZ_2	1.Level, *Wilhelmstehender*	Standing water
RZ_3	1.Level, *Wilhelmstehender*N	Standing water
RZ_4	1.Level, *Wilhelmstehender*N, downAbbaustrecke	Standing water
RZ_5	1.Level, *Wilhelmstehender*N, downAbbaustrecke	Standing water
RZ_6	1.Level, *Richtstrecke Wilhelm-stehenderS*, byth *Bergziege*	SlimySediment
RZ_7	1.Level, *Wilhelmstehender*S, below the hospital of Freiberg	Standing water

Table 1.3: In situ geochemical data in the sampling points.

The samples were brought to the laboratory for filtration as soon as possible while cooling.

4.2 DNA extraction

Filtration

For detection of microbes water samples required a filtration step to first trap and concentrate the organisms.

Samples RZ_3, RZ_6, RZ_7.1 and RZ_7.2 were vacuum filtered (Image 2) first through a 0.45 µm gridded sterile membrane and then through 0.22 µm membrane MicronSepCellulosic (Thomas Scientific Company, New Jersey, United States).

For samples RZ_2. RZ_4 and RZ_5 a Watson-Marlow 323 pump (Image 1) was used running at 200 rpm that allowed to filter directly through a 0.22 µm membrane.

Image 1: Watson-Marlow 323 pump, DNA extraction

Image 2: Vacuum, DNA extraction

The filters, 0.45 and 0.22 µm, were stored inside 2 ml Eppendorf tubes at -80ºC for the following DNA extraction. All filters for the same sample were kept together, using in

some cases more than one Reaction Eppendorf for sample. In order to facilitate the storage membranes were cut into two pieces and rolled before been introduced inside the Reaction Eppendorfs (Image 3).

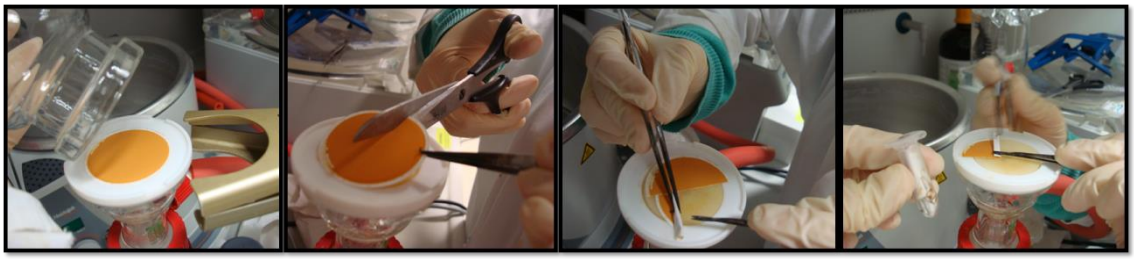


Image 3: membrane storage after filtration, DNA extraction.

Extraction

For DNA extraction from filters two different kits were used: *PowerSoil® DNA Isolation Kit* and *PowerWater® Sterivex™ DNA Isolation Kit*, both from MO BIO Laboratories, Inc (California).

The protocols used for each kit were similar to the recommendations of manufacturer except for some seldom substitutions in the amount of components. These variations were as follows.

a) *PowerSoil® DNA Isolation Kit*
   - The first critical step of the procedure was to cut the filters into small pieces and kept in new sterile Eppendorfs. It was noticed that the smaller pieces, the merrier results in the DNA extraction.
   - 2ndStep; Increase on the incubation time to 1.5 hours
   - 14thStep: Instead 1.2 mL of Solution C4, 500 µL was added.

   The *Alternate Protocol for PowerVac^{TM} Mini Spin Filter Adapter* was not used.

b) *PowerWater® Sterivex™ DNA Isolation Kit*
   VacMaster™ vacuum was employed, providing faster results than *PowerSoil® DNA Isolation Kit*

In order to verify the extraction, a measurement of the DNA concentration was done using NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, United States)

4.3 Cloning by PCR, PCR clean-up and Electrophoresis

PCR

To control a successful extraction of the DNA from the water samples and the PCR run, positive and negative controls were used along with samples. Negative and positive controls were composed by PCR mix (Table 4) without DNA template and distillated water, respectively.

A PCR of samples with specific primers 27F and 1387R (Table 2) for 16S rRNA gene amplification was performed.

Primer	Sequence
27F	5´- AGA GTT TGA TCC TGG CTC AG - 3´
1387R	5´- GGG CGG NGT GTA CAA GGC - 3´

Table2: Sequence of primer 27F and 1387R

Polymerase Chain Reaction with the 16s mode (Table 3 and 4) of samples was run to generate copies of the DNA extracted in a Biometra TGradient Thermocycler (Biometra GmbH Company, Germany). For this PCR a set of 12 samples was prepared: Eight water samples, two controls (negative control plus a positive control) and one additional sample without DNA template in order to correct the possible pipetting mistakes and to secure enough amount for each mini tubes.

Step	Temperature in °C	Time	Repeat
Top heating	94	5min	
Denaturation	98	30 sec	29x
Annealing	50	30 sec	
Elongation	72	30 sec	
Break	15 °C	-	

Table3: Program for 16S-rRNA gene PCR

Components	Approach for 12,5 (µL)	Approach for 25 (µL)	x 12 from 12,5 column (µL)
Master Mix	6,25	12,5	75
BSA	0,0625	0,125	0,75
DMSO	0,625	1,25	7,5
Primer 27f	0,15	0,3	1,8
Primer 1387r	0,15	0,3	1,8
ddH_20	4,26	8,53	51,12
DNA Template	1,0	2,0	1,0 per sample

Table4: Composition of the PCR-mixture for 16S-rRNA gene

Samples that did not give proper results in the analysis were subjected to a new PCR with the intention to increase the DNA concentration up to 50-150 ng before repeat the process. In this case the PCR Dream Taq (ThermoScientific, Molecular Biology, United States)(Table 7) was used in order to overcome the absence of some sample bands after 16s PCR that was noticed by using the standard Master Mix. Dream Taq is an enhanced Taq DNA polymerase optimized for all standard PCR applications and it ensures a higher sensitivity than Maxter Mix (Weyant, R. S. et al., 1990)

PCR-cleaning

After PCR, samples were cleaned up in order to purify PCR products from enzymes, dNTPs, primers and other undesirable reaction by-products. The PCR products cleaning was done using UltraClean® PCR Clean-Up Kit (Mobio laboratories, Carlsbad,

California) according to manufacturer instructions except for steps 4t^{h}, 8th, 10th and 13th which were run at the maximum centrifuge speed and time; and 12th Step that instead 50 µl of Elution Buffer, 35 µL dH_2O was added.

Gel Electrophoresis

After PCR products cleaning a gel electrophoresis was run to determine if the targeted 16S gen was successfully amplified by visualizing within the gel exclusively the size fragments corresponding to 16s rRNA.

For this purpose, 30 mL agarose gel composed by 1 x Buffer TAE at pH 8 (Sambrook et al., 2001) and 1% of Agarose (Biozym, Germany) was used (Table 5). A mixture of PCR-product and loading buffer (10 µL) was loaded in the gel. Also 1 kb-size marker was also loaded in the gel to compare the fragment length of the PCR product. The gel was run at 90 V for 20 min.

After staining with ethidiumbromide (30 seconds) and water (30 min), the gel was documented with the ChemiGenius Bioimaging System (Syngene, Cambridge, United Kingdom) using the Software Genesnap. If the DNA extracted was successfully amplified a PCR- product with a length of 1350 bp could be found and these samples were used for further steps.

TAE	**Amount**
Tris	242 g
Acetic acid	51.1 g
EDTA (0.5 M)	100 mL

Table 5: 1 x Buffer TAE composition

4.4 Microbial composition determination by T-RFLP

16s r-RNA gene PCR

A second 16s PCR amplification in order to reach 150 ng/µl DNA concentration for each clone and label the end restriction sites was run. In this case four repetitions for each sample were carried out. The cleaned PCR products (25 µL) were labelled by using in this case universal fluorescent 27 cy5 PCR primer, instead of primer 27f (Table 2).Once the PCR was run all mini-tubes with the same clone were pooled together.

During the set-up of this step a difficulty appeared: due to the fluorescent characteristic, primer 27 cy5 cannot be exposed to the light, neither the samples containing it. In order to overcome the difficulty and secure an adequate process, the samples were covered with black plastics thus avoiding the light inside the tubes and painstakingly handled.

Gel electrophoresis and PCR-cleaning

The same methodology than previously described for gel electrophoresis was employed (Materials and methods, Cloning by PCR, PCR clean-up and Electrophoresis, page 16-17) but 3 µL Fluorescent PCR product instead 8 µL was loaded. The procedure was done as described in the UltraClean® PCR Clean-up Kit Protocol (MO BIO Laboratories Inc., California) but DNA was dissolved in 30 µL dH2O instead Elution Buffer. As a process assessment, the DNA concentration was measured by NanoDrop® ND-1000 Spectrophotometer

Digestion

Digestion process consisted of cutting amplified DNA at specific sites. Such cuttings were performed by restriction enzymes MspI (BioLabs Inc., New England) and AluI (ThermoScientific, Unites States) according to the reaction mixtures reported in Table 6.

Mixture	Amount	Type
Buffer	1.0 µL	Buffer R (ThermoScientific) for AluI
		SmartCut (BioLabs Inc) for MspI
Restriction Enzymes	0.1 µL	AluI for all samples
		Msp I for RZ_4.2 and RZ_6
DNA	150 ng	
d H_2O	Up to 10 µL	

Table 6: Digestion mixture for T-RFLP

Once the mixture was prepared it was incubated during 3 h at 37ºC and afterwards kept at -20ºC until the following T-RFLP analysis.

T-RFLP

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis can be considered one of the most relevant steps in this research. This is because the technique allows determining the microbial community composition, i.e, the biodiversity of the different water samples gathered in Reiche Zeche, by detecting the restriction site closest to the 27 cy5 labelled end of the enzyme digested DNA.

CEQ™ 8000 Series Genetic Analysis System (Beckman Coulter Life Science Researches, Barcelona, Spain) was used to run the T-RFLP. This equipment contains diverse trays, which were filled with the mix described in Table 7.

Components	Amount
Buffer Genome Separation Buffer	More than the half directly inside each well.
Fluorescent SLS+ standart	28.5 µL
DigestedSample	1.5 µL

Table 7: T-RFLP mixture

It is important to remove air bubbles from each well before running the T-RFLP and overlay them with one drop of mineral oil in order to avoid evaporation.

New PCRs and new T-RFLP

Samples that did not give proper results, as for example sample RZ_5, in the T-RFLP analysis previously described, were subjected to a new PCR with Dream Taq (Table 8) (*Cloning by PCR, PCR clean-up and Electrophoresis*, page 18).

PCR Dream Taq		
Mixture	**25 µL column**	**x 8**
Dream Taq	0.25 µL	2 µL
dNTP coach	2.5 µL	20 µL
Primer 27f	0.3 µL	2.4 µL
Primer 1387f	0.3 µL	2.4 µL
Buffer	2.5 µL	20 µL
dH_2O	17.15 µL	137.2 µL
DNA Template	2 µL	2 µL

Table 8: 16s Dream Taq PCR mixture

Succeeding the new T-RFLP analysis, Sample RZ_5 and RZ_6 were selected for sequencing (Results: T-RFLP).
Regarding to the last PCR results, it was proven that sample RZ_6 was more efficiently amplified with Master Mix, meanwhile sample RZ_5 was properly amplified using only Dream Taq.

4.5 Cloning

The extracted DNA (*DNA extraction*, page 15-16) was cloned into *E. coli* in order to get a clone library of bacteria from water samples. This process required three steps: ligation, transformation and cultivation.

Ligation

The StrataClone™ PCR Cloning Kit (Agilent Technologies, Canada and United States) was used for ligation according to manual instructions. The mixture reported in Table 9 containing the plasmid used as a vector, buffer and PCR product (RZ_5 and RZ_6) was incubated for 5 min at room temperature and afterwards placed on ice to stop the reaction.

Mixture (for sample)	Amount for sample (µL)
StrataCloneTM Cloning buffer	3
Clean 16s PCR-product (50 ng/µL)	2
StrataCloneTM Vektor Mix Amplifican	1

Table 9: Ligation mixture, regarding with StrataCloneTM PCR Cloning Kit

As a result of ligation, PCR product inserts into operon Lac site of plasmid (Figure 3).

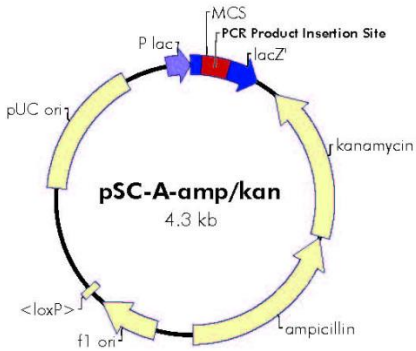


Figure 3: Vector pSC-A (Agilent technologies, Canada, Unite States).

Transformation

According to the instructions of the StrataClone™ SoloPack Competent Cells Kit (Agilent Technologies, Canada and United States) 2 µL of ligation mix was added into a tube of *E. coli* competent cells and incubated on ice approximately for 20 minutes. This technique requires that the host cells are exposed to an environmental change which makes them "competent" or temporarily permeable to the vector. That is why the total mixture was warmed up to 42ºC during 45 s, generating a transformation by heat shock. The heat shocks must be rapidly stopped by incubation on ice for 2 minutes.

Summarizing, in this process *E.coli*'s vector pSC-A is cut. This cutting site is located between a section of DNA with numerous restriction cutting sites (multiple cloning site) and lacZ-gene. It is there where the fragment of the 16S-rRNA gene is incorporated.

Cultivation

LB_ampicillin medium (Sambrook et al., 2001) was chosen for carrying cells cultivation (Table 10).

Components	Amount
Trypton	8g
yeast-extract	4g
NaCl	8g
Agar-Agar for solid medium	13g
dH_2O	800mL

Table10: LB_ampicillin medium

The medium was adjusted between 7.0 and 7.2 pH with 1N NaOH, autoclaved and cool down up to 55ºC. After reaching this temperature 0.8 mL Ampicillin 100µg/mL were added and the medium was poured on Petri plates.

Next in the line, eight diverse cell culture dilutions or transformation product, with different concentration per sample (RZ_5 and R_6) were poured on the Petri plates (Table 11). Sometimes liquid LB medium was also added (Table 11) to facilitate the platting, in those who did not have sufficient cell mix amount for been spread (considering that start to be enough amount with 30 µL) and finally the plates were incubated at 37ºC.

Plate number	[cells mix] µL	liquid LB medium
1	5	55
2	5	55
3	20	40
4	20	40
5	50	-
6	50	-
7	100	-
8	30	-

Table 11: Transformation product and liquid LB medium amount on the Petri Plates.

Summarizing, sixteen plates were incubated overnight, eight from RZ_5 and the same number from RZ_6.

Just after, the outgrowth period, ten LB_ampicillin_plates were selected for being spread with 40 µL X-galon each plate for blue-white colour screening. This is because StrataClone cells strain has a mutation which supports dark blue colour when it has no

absorbed the plasmid, therefore colonies harbouring plasmids containing typical PCR product inserts are expected to be white or light blue after prolonged incubation facilitating the identification of the colonies with the plasmid of interest inside.

4.6 ARDRA

With a view to identify the gene sequences and the microorganisms' fingerprints, ARDRA technique was chosen. ARDRA consisted of the 16s amplification and in the subsequent digestion of the amplification product with enzymes and separation by electrophoresis (Blaszczyk, 2011). The fragments obtained after enzyme digestion were separated by electrophoresis in agarose gel according to their size. Therefore, fragments created a vertical line (from negative to positive in the agarose gel) with several bands. Such lines were considered as the clones' "fingerprint".

However, first of all a selection of clones, their numeration and a PCR with the primers T3 and T7 was necessary.

Clones Selection and T3T7 amplification

Two hundred and twenty mini tubes for each sample were prepared for T3T7 PCR with the components from the column 15 µL (Table 12).

Mixture	Approach for 15 µL	x 226 per sample (µL)
2 x MM	7.5	1695
T3	0.18	40.68
T7	0.18	40.68
DMSO	0.38	85.88
BSA	0.04	9.04
dH_2O	6.72	1518.72

Table 12: T3T7 PCR mixtures

This step tries to focus its activity on the separation of the *E. coli* colonies with insert. For that all white colonies were picked with sterilised picks from the plates with different cell mix concentrations, and transferred on a fresh and numbered LB_plates (Image 3) with 40 µl 2% X-Gal already on them. With the same pick per clone, was also placed a little amount inside the mini tubes for T3T7 PCR.

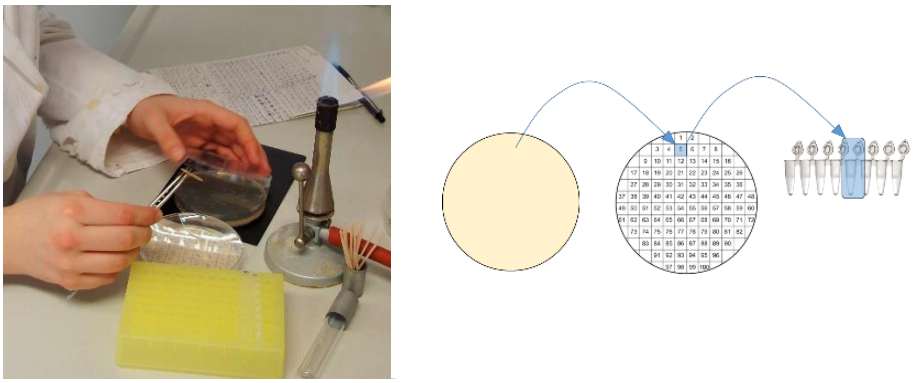


Image 4: Colonies separation and T3T7 preparation

Digestion

After amplification T3T7 PCR products were subjected to an digestion with BsuRI enzyme (Table 13). BsurI restriction enzyme (ThermoScientific, United States) had the responsibility in this case to recognize determinate parts of the gen and cut them with high yields at 37ºC (tiempo?).

Mixture	Amount per clone (µL)
dH2O	4.9
Buffer R	1
T3T7 PCR Product	4
Enzyme BsuRI	0.1

Table 13: Digestion mixture for ARDRA

The visualization by the electrophoresis gel of the digestion product brought to us pictures with a big range of shapes, where each different shape belongs to a one specific species (Images 6 to 22). With a simple but painstaking gathering of the shapes it is possible to get an approach about diversity and population, similar than the one obtained with the T-RFLP analysis.

4.7 Clones Storage

The Storage of clones will allow further investigations to continue the research, considering that it has, as main objective, the preservation of the microorganisms under laboratory conditions, maintaining their vitality and avoiding spontaneous mutations (Donev, 2001) thus enabling future uses of the samples.

In this research the Cryobiological Method (Donev, 2001) was used for clones storage. This was performed as follows.

First one glass tubes per clone with 3mL liquid LB_medium and 0.3 µL ampicillin were prepared. Working in a sterile atmosphere, the numerated colonies from the numerated Petri Plates (*Clones Selection and T3T7 amplification*, page. 23) were transferred to the

liquid LB_medium. After an overnight incubation at 37º, the presence of turbidity or pellets at the bottom of the liquid LB_medium tubes was indicative of a positive growth of *E.coli* with plasmid.

For the next step in the storage, 700µL liquid LB medium with positive clone reproduction were transferred into 2µL sterile reaction plastic tubes, named with the corresponding sample (RZ_5 or RZ_6) and clone number. Moreover, 700µL prewarmed glycerine was pipetted into the same reaction tubes and strongly vortexed looking for a homogenous mixture.

Reaction tubes were finally placed inside gridded boxes and rapidly frozen employing liquid nitrogen. With this fast freezing the process of recrystallization of the ice is prevented and mechanical membrane damages are avoided (Donev, 2001). Finally all pre-frozen clones were stored at -80ºC.

4.8 Sequencing, cultures identification

The sequencing itself was carried out by an external laboratory GATC Biotech AG (Konstanz, Germany). The unique requirement for the correct analysis was to send fresh cultures on a tray. In order to get these fresh cultures, LB_plates with X-gal were newly prepared and a small amount of the mix kept inside the plastic reaction tubes stored at -80ºC were spread and incubated overnight. Only the most relevant clones for each ARDRA group were cultivated for the sequencing (Table 14)

Sample	Clone
RZ_5	6, 66, 62 68, 150, 79, 103, 20, 14, 24, 105, 22, 15, 11, 54, 88, 217, 27, 32, 12, 122, 52, 101, 17, 26, 133, 168, 18, 28, 33.
RZ_6	10, 126, 9, 12, 15, 81, 72, 198, 149, 53, 31, 39, 41, 42, 50, 60, 63.

Table 14: most representative clones of each ARDRA group selected for sequencing.

The forward and reverse sequences obtained from each clone were edited, aligned and assembled in a fasta-file by using the StanPackage.

Next in the line, the tool BLAST of the NCBI-Database was used in order to find relatives or similarities to other species with the created fasta-file. The phylogenetic treewas created with the NCBI_Database results by using the program MEGA6 with the Maximum Likelihood method (Image 22).

4.9 Clone Library

This last task looked for attach the results of the cloning experiment to the Clone Library. T-RFLP analysis on each single clone was required, called T-RF. By the reason to figure out the clones' identification and fill with such information the Clone library with the clones 'information (McClean, 1997).

Therefore inside this data base, saved information from each ARDRA representative clones identified is stored for further possible uses.

Individual T-RFs allowed not only validate each of the peaks of the original T-RFLP, also related T-RF "assessed the relative abundance of each variant in the library either"

For such T-RF analysis is required to have directly access to the genetic material or plasmid. Consequently in order to perform T-RF it was necessary a first clone DNA extraction from the *E.coli* cell.

Plasmid extraction

For the clones' DNA extraction, Thermo Scientific GeneJET™ Plasmid Miniprep Kit was used. According to instructions attached in the Kit, before setting up the extraction, new tubes with 3mL liquid LB_medium were prepared and cultivated with the representative clones coming from the fresh plates used to sequencing for a next overnight incubation.

In general, after several reactions between cultivated liquid LB_medium and lysis solutions and wash solutions, plasmids DNA wereliberated from recombinant *E. coli* cultures.

T-RF

T-RF and T-TRFLP techniques are similar, thus, as it was described before (*Microbial composition determination by T.*RFLP, page 18) the analysis was made up by the same four steps, except for substitutions in the AluI restriction enzyme by BsurI and the following Buffer R.

5. Results and Discussion

5.1 Water Sampling, *in situ* geochemical data.

A low or negative ferrous iron presence together with a high ferric iron presence (Reaction 3) and elevated redox potential are indicators of iron oxidizer activity. Thus it was expected to find microorganisms related to this activity in the samples RZ_2 RZ_5 and RZ_6.

The pH ranged between 6.5 and 2, proving the permanently acid condition of the mine water (Table 1.2).

Low parameters were in general detected for the sample RZ_1, even the ferric concentration. These results can be explained by the sampling point. RZ_1 was collected from the mine roof and no puddling water

5.2 DNA Extraction

It is notable the difference between concentrations depending on the Kit employed. Indeed the origin of these differences was the improper handling of the membranes. PowerWater® Sterivex™ vacuum was run up to high pressures, overcoming the membrane pressure limit and developing cracks on the filter.

PowerWater® Sterivex™	[DNA] ng/µl
RZ_1	4.8
RZ_ 2.2	5
RZ_ 4.1	3.2
RZ_ 4.2	2.4
RZ_ 5	4.0

PowerSoil®	[DNA] ng/µl
RZ_3	42.4
RZ_6	9.4
RZ_7.1	5.6
RZ_7.2	7.0

Table 15: First DNA concentration analysis with NanoDrop® ND-1000 Spectrophotometer

5.3 PCR

Several 16sPCR were run. Image 5 shows one example of 16sPCR visualization by agarose gel. In this example wrong results, marked with a square, and right results after PCR were obtained. Those with wrong results were repeated. Only sample RZ_6 needed the application of Dream Taq

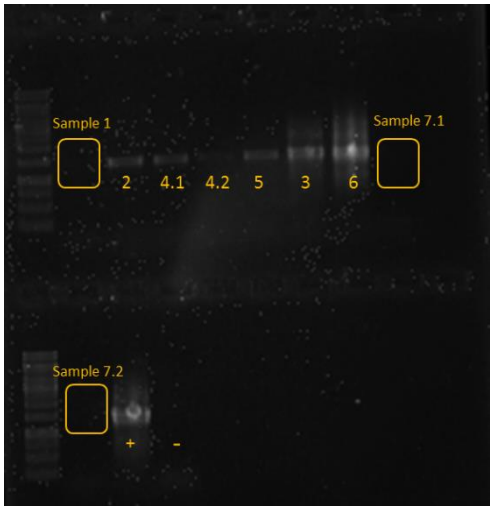
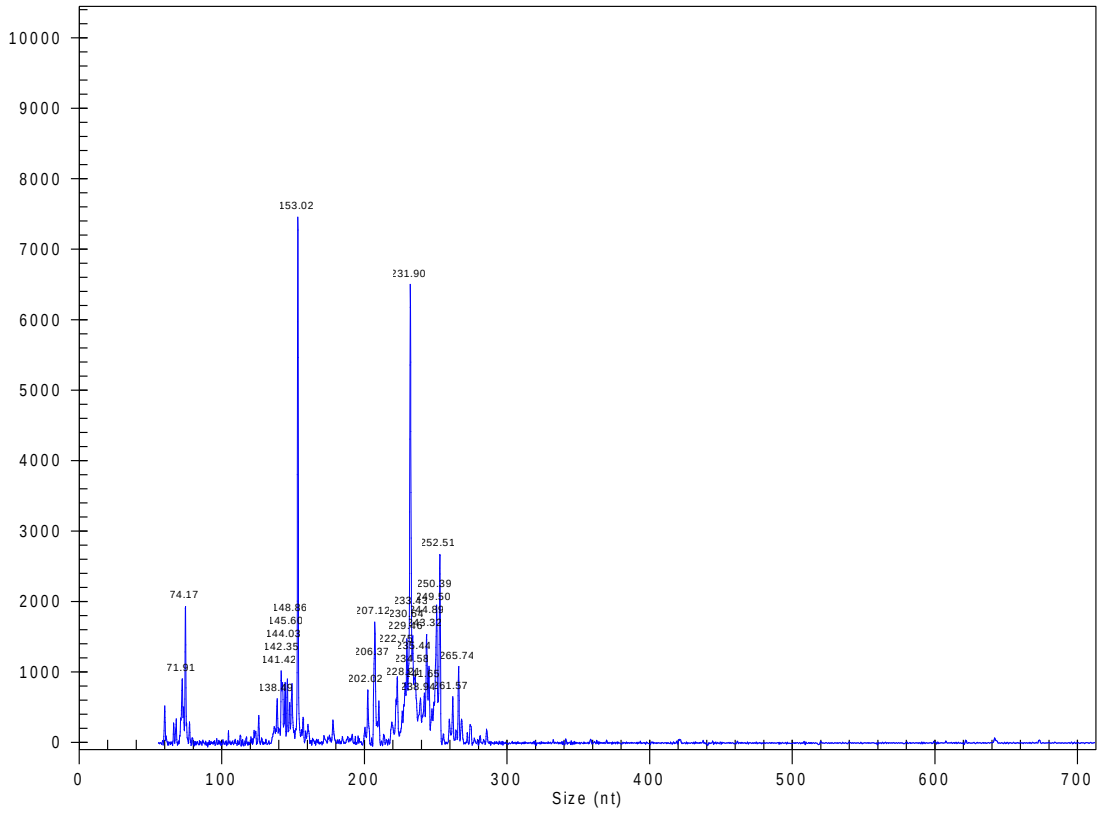


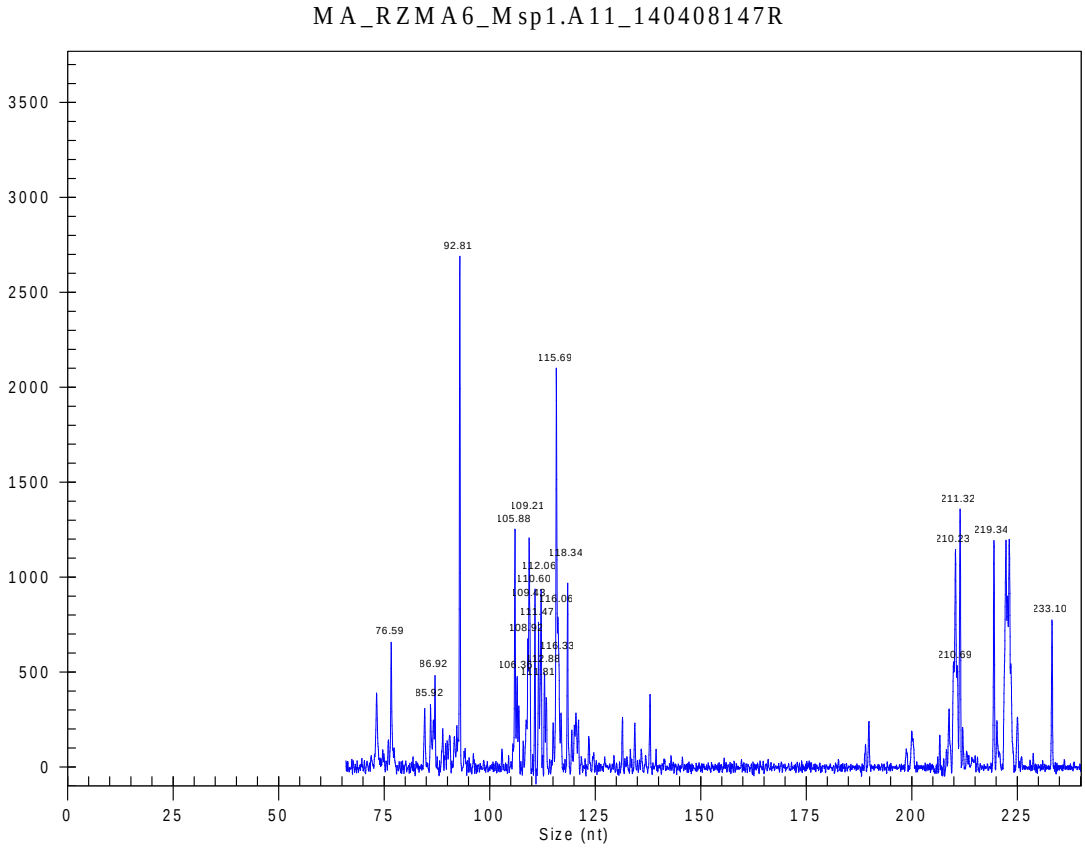
Image 5: Result of PCR product and controls in a gel electrophoresis.

5.4 T-RFLP for diversity

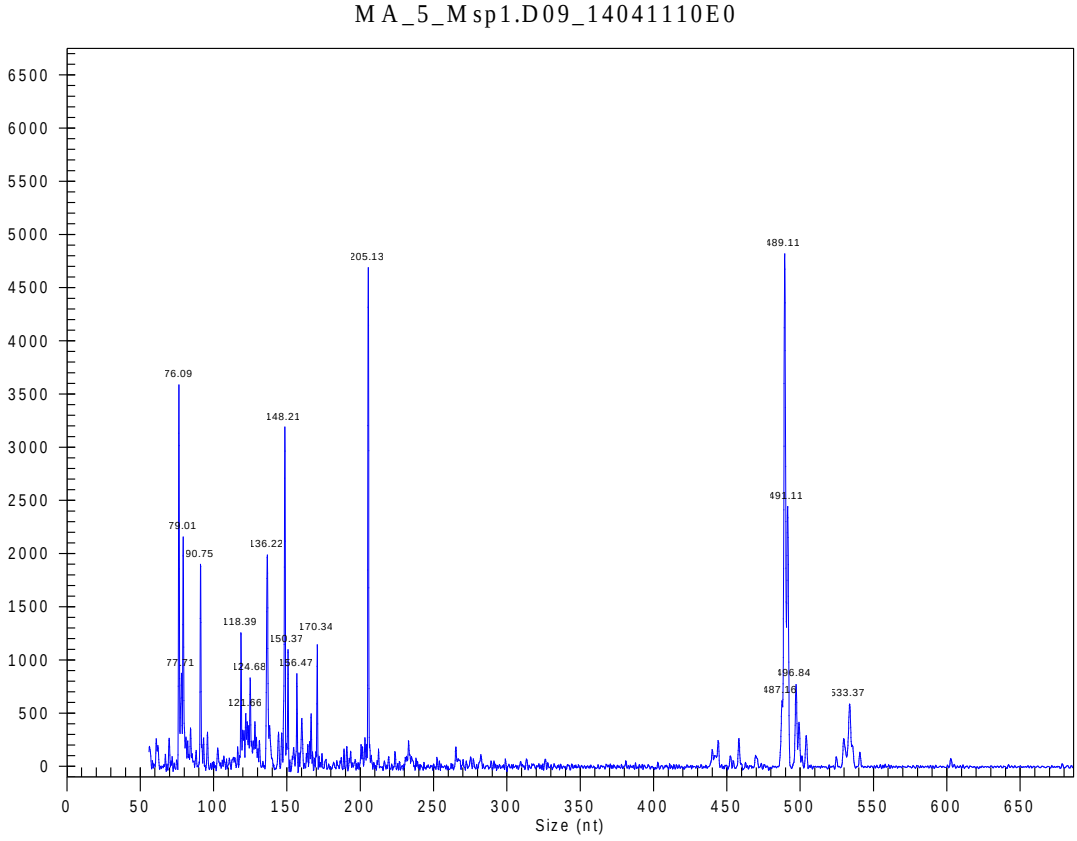
Resulting T-RFLP graphics, also named electropherograms, described the communities' diversity assessment. Such electropherograms have provided information on a collection of microorganisms that might be present in the different samples. Series of peaks (DNA fragments) of various heights represents the profiles of each samples. Each peak corresponds to one genetic variant in the original sample while its height corresponds to its relative abundance in the specific community (Osborn, M., 2000).



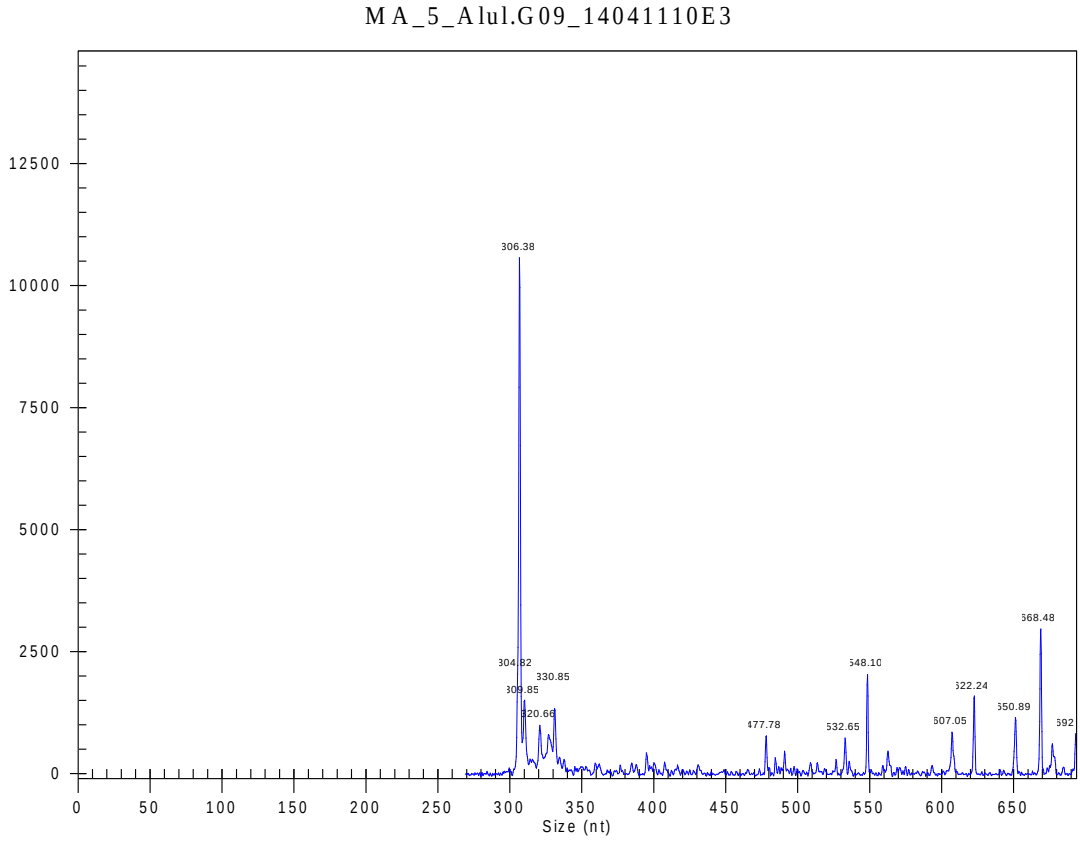
Graphic 1: RZ_6 T-RFLP results with AluI enzyme



Graphic 2: RZ_6 T-RFLP results with MspI enzyme



Graphic 3: RZ_5 T-RFLP results with MspI enzyme



Graphic 4: RZ_5 T-RFLP results with AluI enzyme

Regarding to the graphics or electropherograms, samples RZ_5 and RZ_6 are examples for the highest diversity together with RZ_2. Around eighteen different DNAs are represented in the graphics for RZ_5 (Graphic 3 and 4) and twenty-two to twenty-eight approximately for RZ_6 (Graphic 1 and 2).

SAMPLE	[mV]±50mV Redox	[Fe3+] mg/L	Fe2+ presence	Number of pics (Diversity)	pH
RZ_1	210	2	-	-	6,5
RZ_2	600	245	Positive	24-26	2 ~ 3
RZ_3	640	4114	Positive	8	2 ~ 3
RZ_4	730	4055	-	6-10	2 ~ 3
RZ_5	**800**	**2576**	**-**	**13-18**	**2 ~ 3**
RZ_6		**9**	**-**	**20-28**	**6,5**
RZ_7	710	7696	-	5-10	2 ~ 3

Table 16:*In situ* geochemical data and diversity

RZ_7 had high metal concentration. Elevated redox potential and a biodiversity approximately between 5 and 10 species. These parameters represent hints of a very active bioleaching in an extremophile medium. However, this sample was not chosen for further analyse because of the diversity results were not high enough.

On the other hand, samples RZ_5 and RZ_6 were chosen for a deeper study because the following reasons:

The relation high ferric presence and low ferrous concentration iron (Reaction 3) matched with the iron oxidizers' activities and the corresponding elevated redox potential obtained from the ferrous oxidation. Both of the samples were found in acid pH and, the most determinant, with high diversity results.

In addition, similar parameters as in the sample RZ_5 were already studied before (by the Bio SciencesGroup, Technic University of Freiberg, Germany) with positive result in iron oxidizer presence. Thus, it was already known that they would be find in this kind of water.

Furthermore, the election of RZ_6 instead RZ_2 was because even both met the characteristics for an iron oxidizer habitat, only RZ_6 had a different acid pH thanRZ_5. It provides the possibility to study a wider environmental range and therefore far greater diversity.

Consequently, samples RZ_5 and RZ_6 were used for ARDRA analysis.

5.5 ARDRA

ARDRA gels provided the protection of the DNA fragments as well as the T-RFLP did. Nevertheless, instead picks ARDRA technique projects the fragments information as bands. Each different DNA is defined on the gels by a collection of vertical bands (Image 6 to 22). Therefore such bands creates several shapes also named fingerprints.

Sample RZ_5

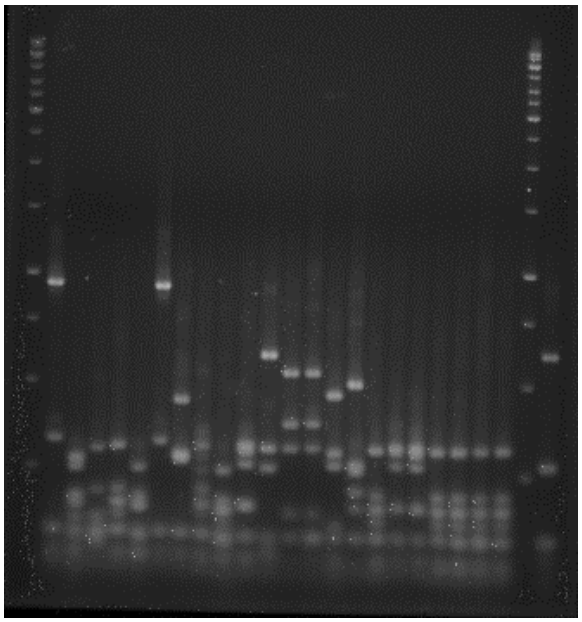


Image 6: gel1_20140526

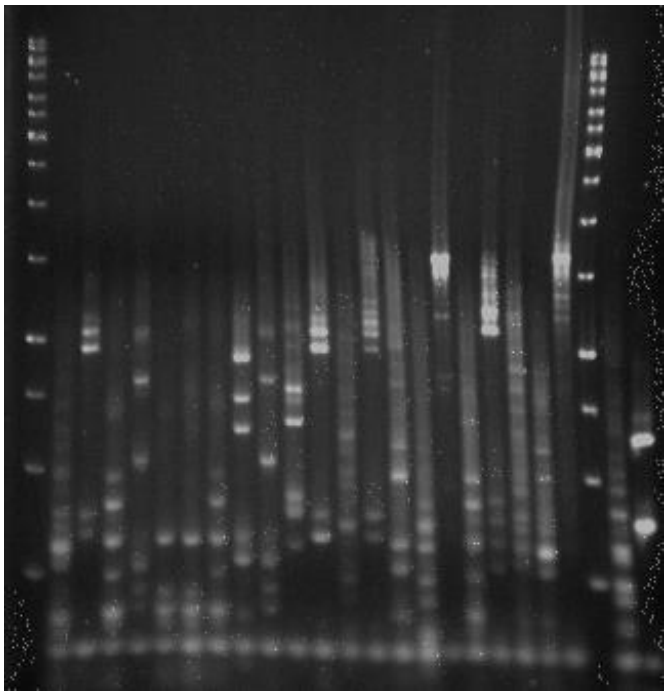


Image 7: gel2_20140526

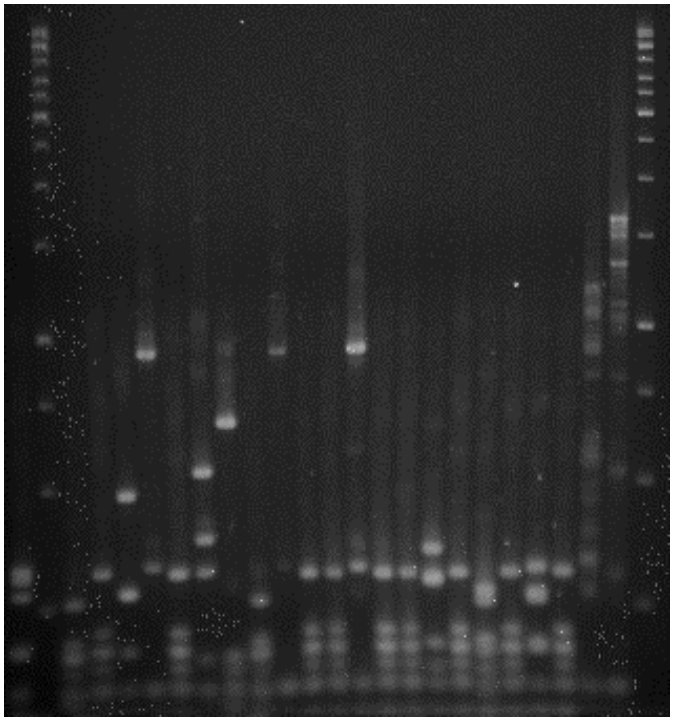


Image 8: gel1_20140527

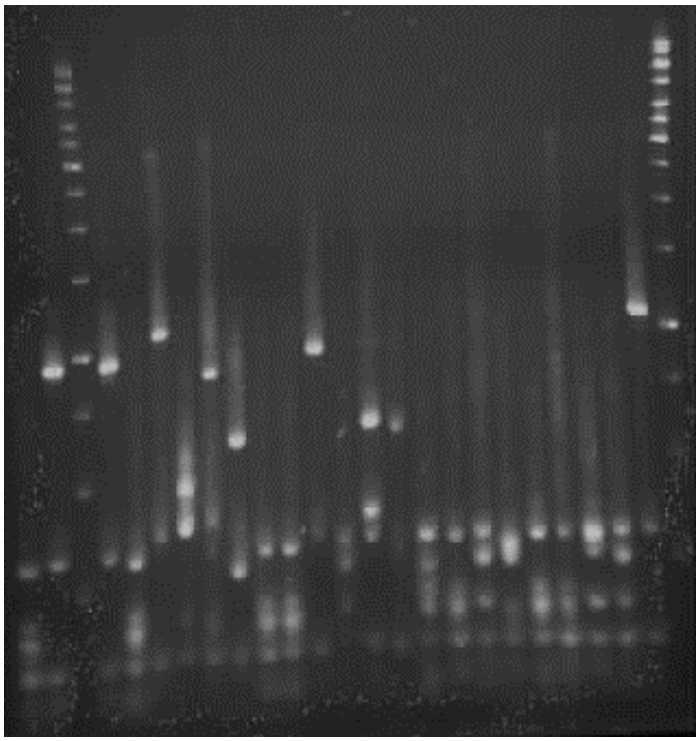


Image 9: gel2_20140527

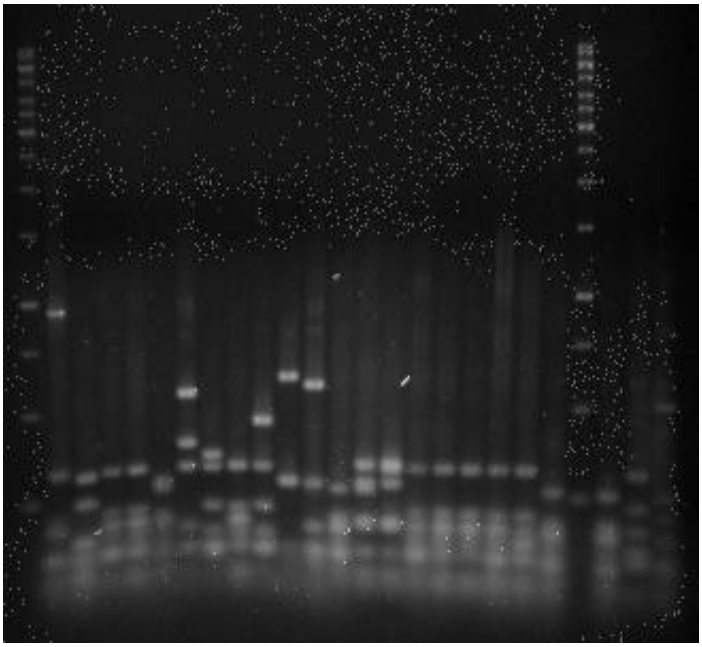


Image 10: gel3_20140527

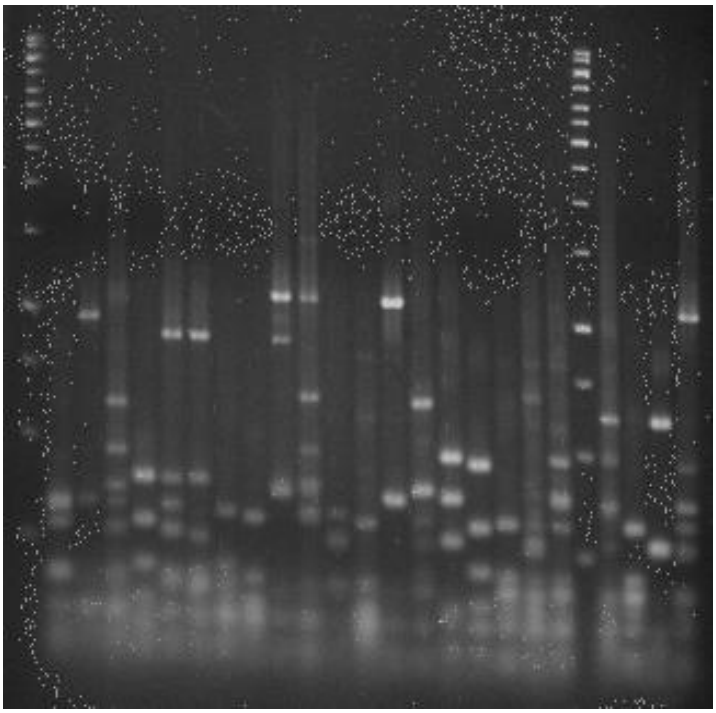


Image 11: gel4_20140527

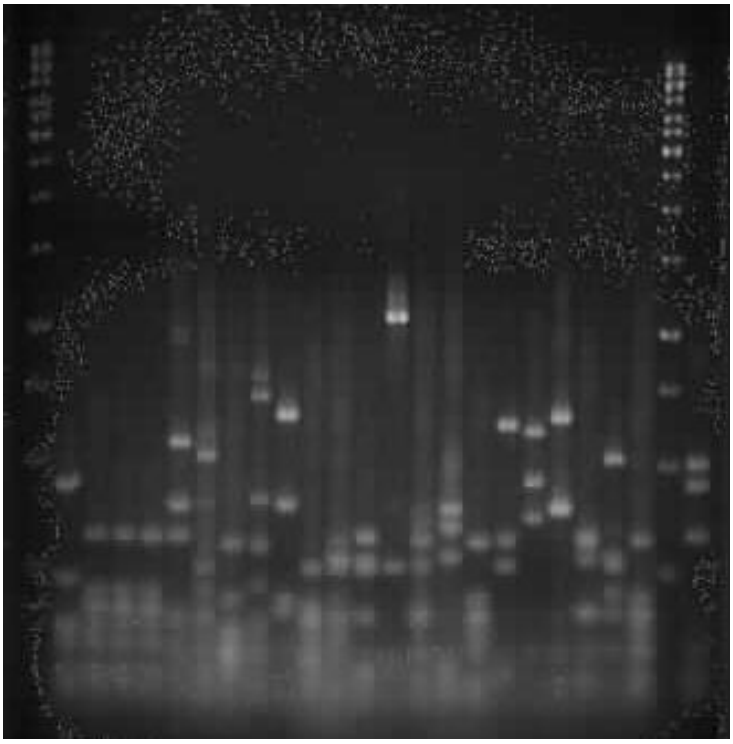


Image 12: gel1_20140530

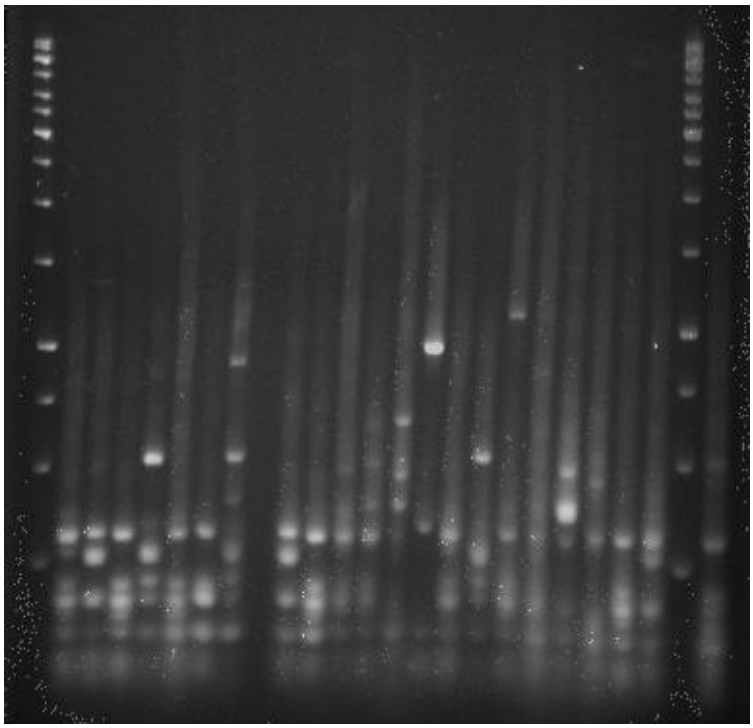


Image 13: gel1_20140617

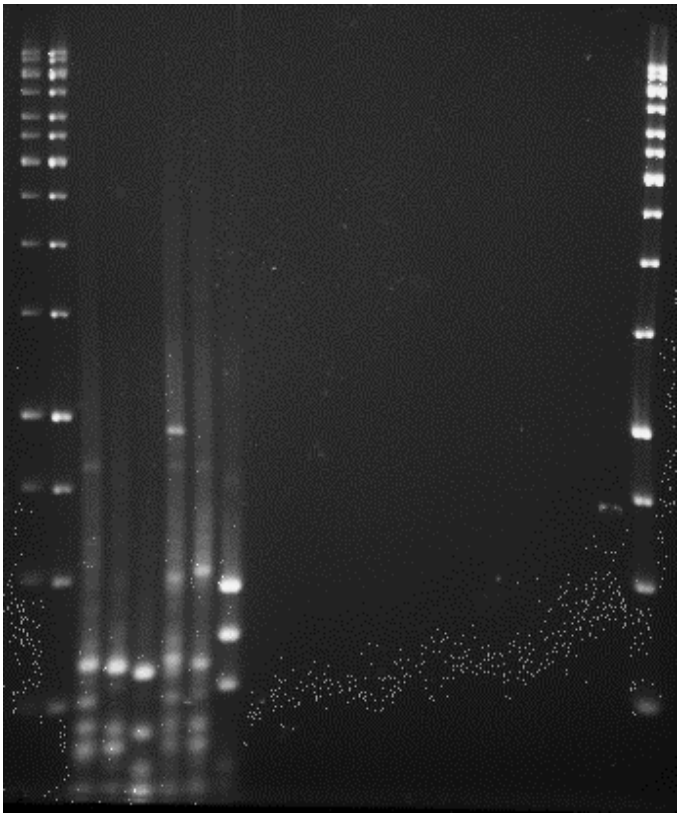


Image 14: gel2_20140617

Image 15: gel1_20140618

Sample RZ_6

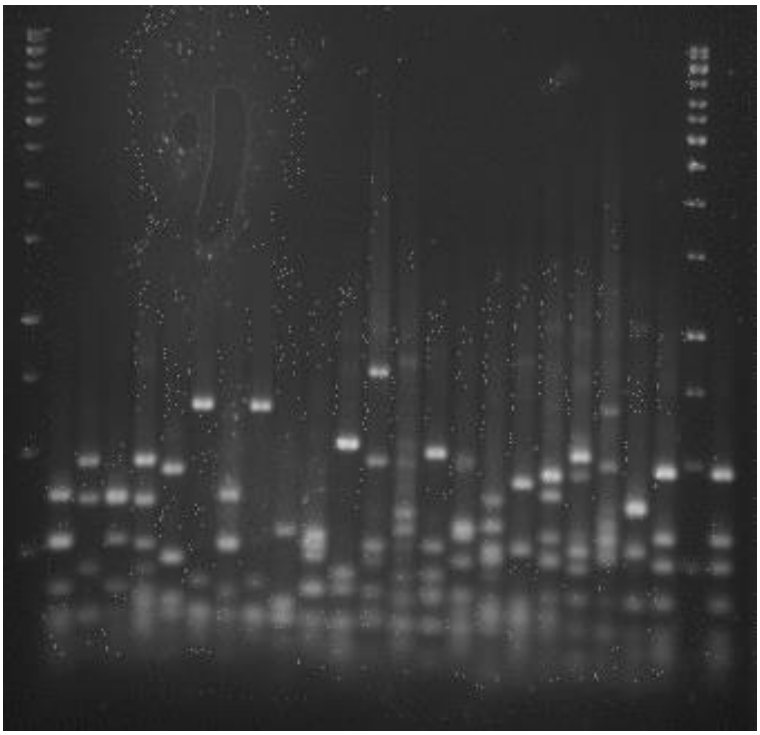


Image 16: gel1_20140424

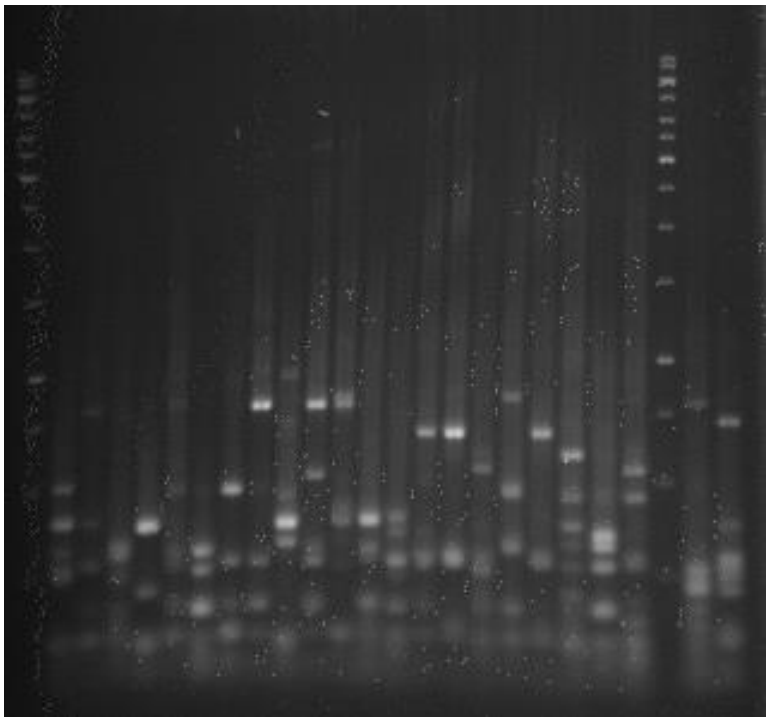


Image 17: gel2_20140424

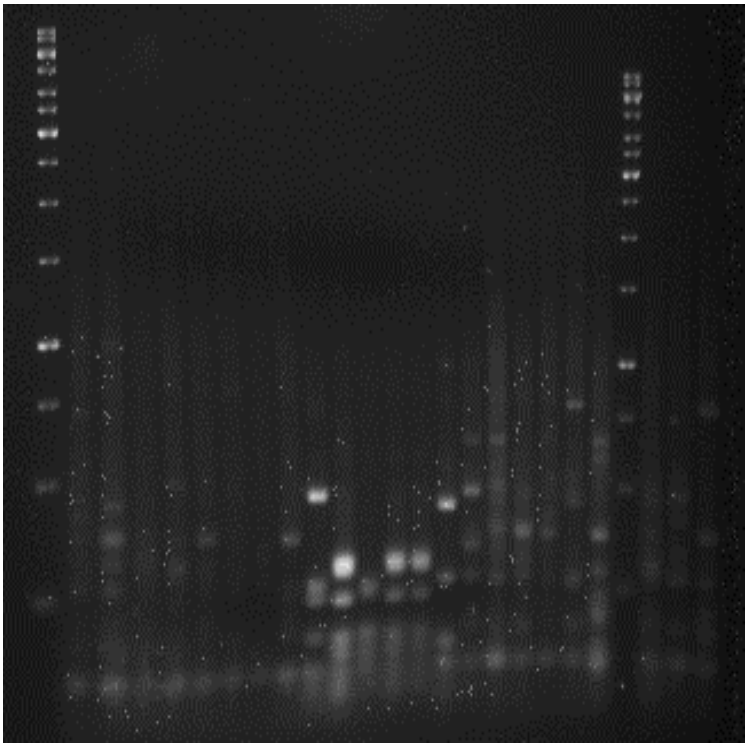


Image 18: gel1_20140425

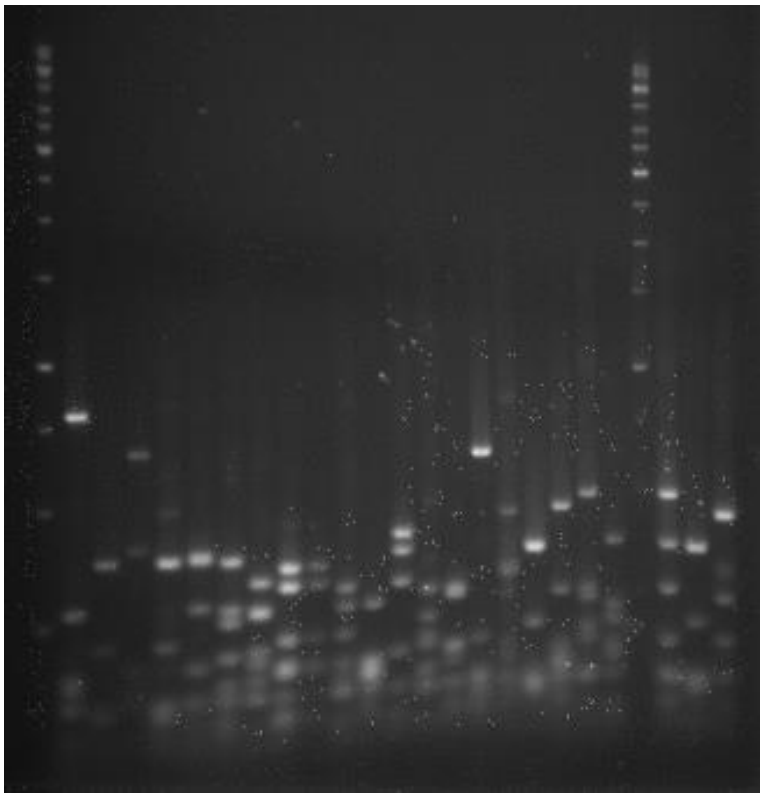


Image 19: gel1_20140428

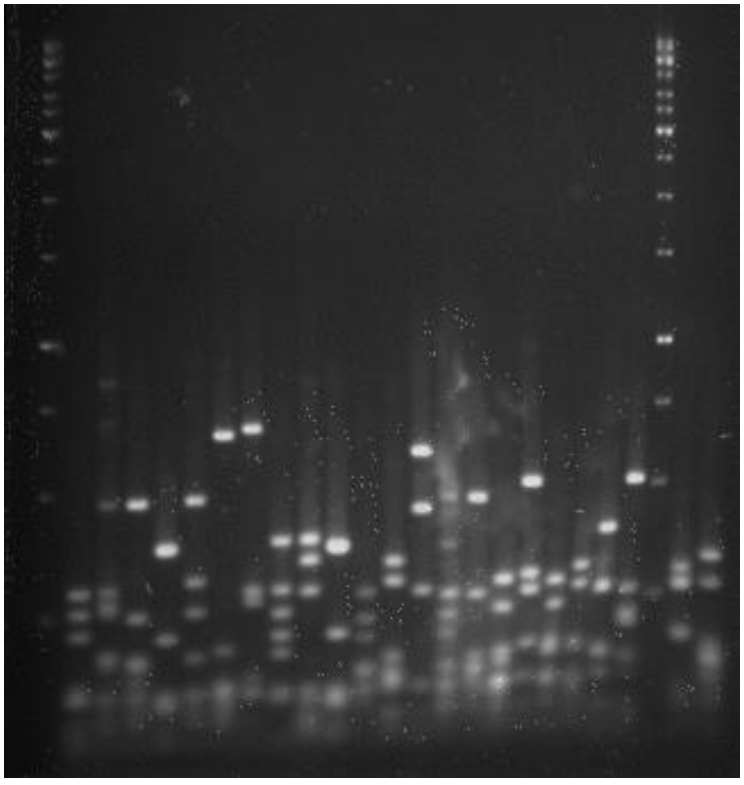


Image 20: gel2_20140428

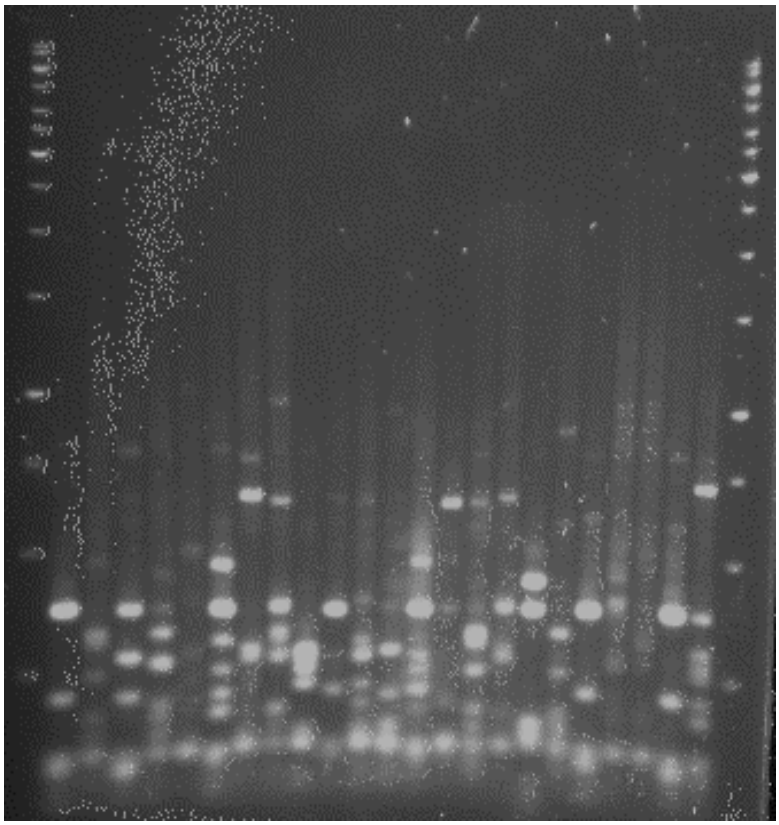


Image 21: gel1_20140502

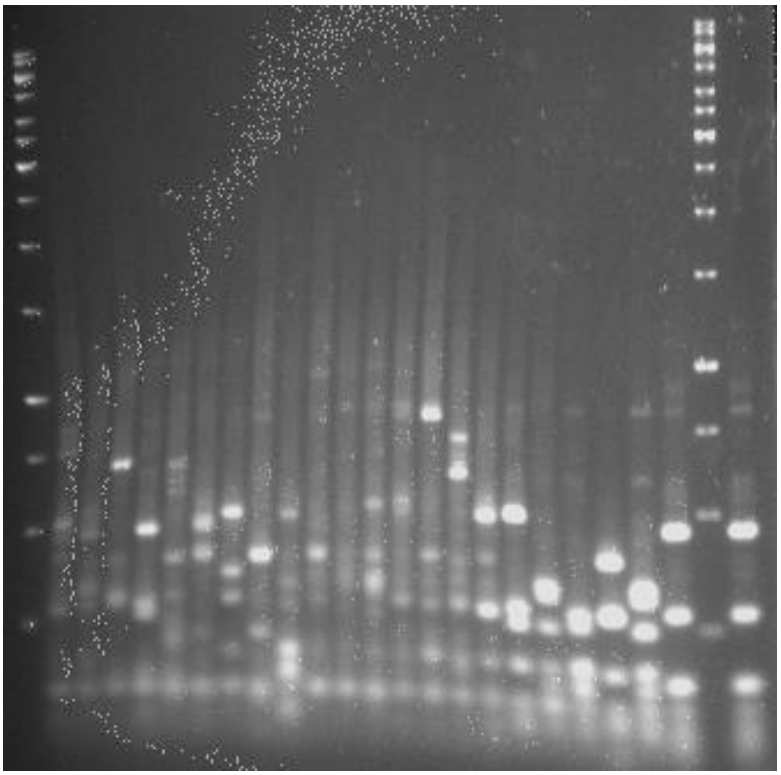


Image 22: gel2_20140502

In total gathering the different shapes or fingerprints, RZ_5 reached the twenty-two groups plus 3 isolated shapes that did not met and with other groups. On the other hand, for RZ_6 were found twenty-six groups plus 7 isolated and unique DNA's shapes.

5.6 Identification of cultures (sequencing analysis)

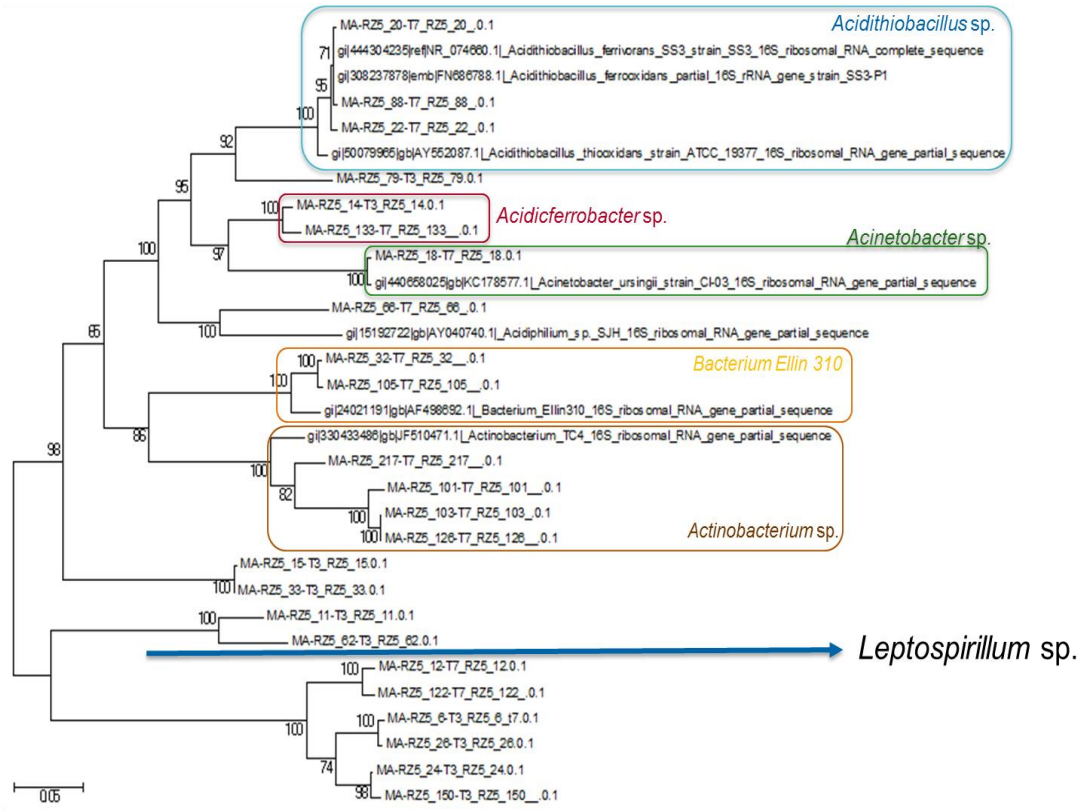


Image 22: Phylogenetic tree showing the different iron oxidizers found in sample RZ_5

Six kind of Iron oxidizer were found in the water samples RZ_5 of *Reiche Zeche* mine: *Acidithiobacillus* sp., *Acidicferrobacter* sp., *Acinetobacter* sp., the soil *Bacterium Ellin* 310(Sait et al., 2002), *Actinobacterium* sp., *Leptospirillum*sp. and many uncultured bacterium large list of uncultivated microorganism which made up their own isolated group in the phylogenetic tree, as for example the last group down to *Leptospirillum* sp.

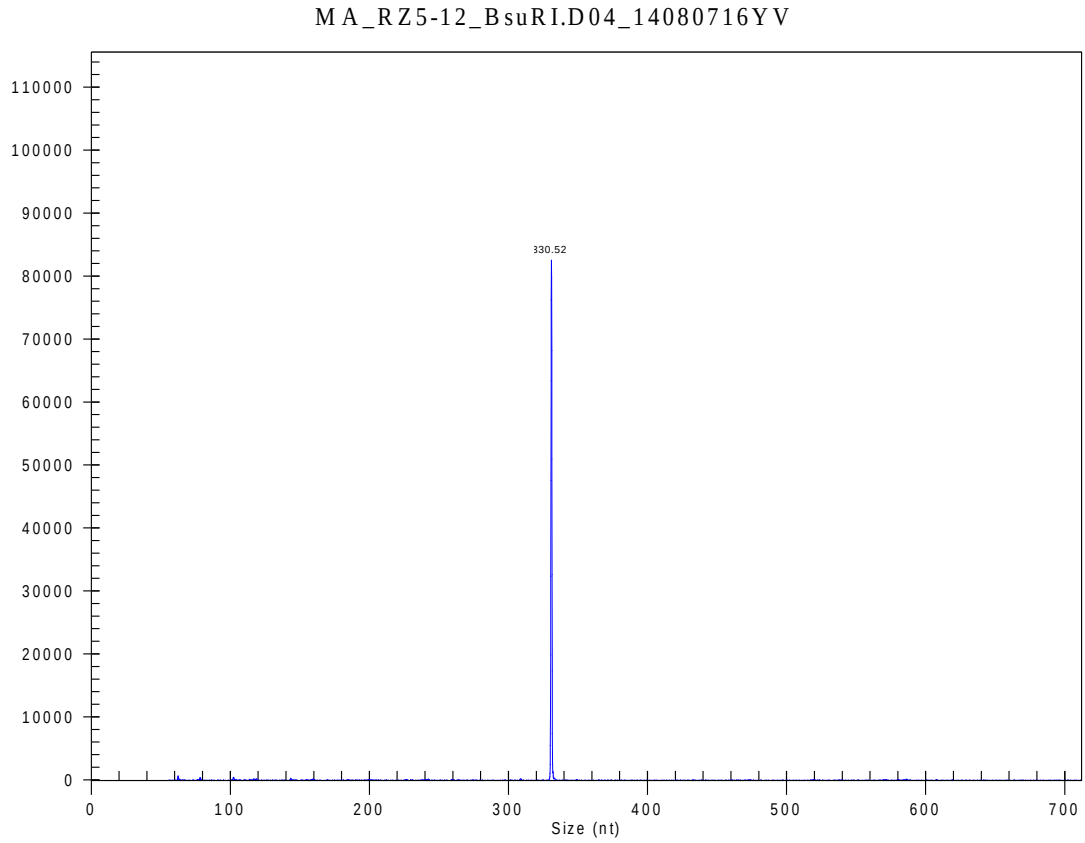
The presence of uncultured bacterium was not considered possible mistake because of the information found in NCBI data base, which described the uncultured bacterium with the phrase: "microbial stratification in low pH oxic and suboxic macroscopic growths along an acid mine drainage". Consequently, NCBI data base confirmed and agreed the acid water mine uncultured bacterium origin.

In addition to aforementioned information, the phylogenetic tree results were also considered successful due to the overall presence of more than 70% of correlation.

RZ_6 sequencing results are unfortunately not available so far, because of complications with the operation time at the University of Freiberg (Germany), limited to six months.

5.7 Clone Library, T-RF

Individual T-RF profiles, with one unique pick, for each organism were obtained as it is shown on Graphic 5. Such profiles helped to identify the different microorganisms which belonged to the sample RZ_5. Information extracted from this process was used to create the clone library.



Graphic 5: T_RF example for a Finger-print of a single clone (Clone12) belonging to the sample RZ_5.

6. Conclusion

The final identification proved the presence of *Acidithiobacillus* sp., *Acidicferrobacter* sp., *Acinetobacter* sp., *Bacterium Ellin* 310,*Actinobacterium* sp. and *Leptospirillum* sp. in the sample RZ_5.

In addition, uncultured bacterium common to acid mine drainage inhabitants were also identified together with the aforementioned iron oxidizers. Such uncultured group made up an independent group.

It is possible that the answer for the favourable geochemical conditions were in the RZ_7, which represents the more efficient bioleaching process of the samplings points. Under this suppose the favourable conditions would be a 2-3 pH range, 11-12 ºC temperature condition, around 24 µS/cm conductivity and 59% Oxygen saturation.

7. Outlook

Uncultured bacterium group reach a very interesting position in the phylogenetic tree because of several reasons: such group is totally independent from to the rest of the groups. In fact, the uncultured group was placed even far from the uppers group, but for a better view the tree and a higher understanding of the phylogenetic the position was modified. Nevertheless the similarity is greater with *Leptospirillum* sp., but there are still considerable differences in between the groups.

Therefore and concluding, further studies on the unidentified bacterium as well as on RZ_7 would be highly interesting with the view to create a representative and completed phylogenetic bioleaching tree and to ascertain the favourable conditions for a high bioleaching yield.

8. References

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